

## REVIEW ARTICLE

# Pediatric Cardiology in the Genomic Era

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While congenital heart disease, cardiomyopathy, arrhythmias and acquired cardiac diseases are common causes of mortality and morbidity in infants and children, the basic underlying mechanisms of many specific pediatric cardiovascular diseases still remains undetermined. Breakthroughs in molecular genetic technology have just begun to be applied in pediatric cardiology stemming from the use of chromosomal mapping and the identification of genes involved in both the primary etiology and as significant risk factors in the development of cardiac and vascular abnormalities. This review will focus on information obtained thus far by molecular genetic analysis in the diagnosis, treatment and overall understanding of pediatric cardiovascular disease pathogenesis examining both the more prevalent congenital/inherited heart defects, arrhythmias and cardiomyopathies, as well as sporadic and acquired disorders. In addition, a survey of the pediatric cardiologist's armamentarium with regards to molecular and genetic analysis is presented highlighting the current use of molecular diagnostic methods including microarray, gene-mapping, proteomic, transgenic and stem cell technologies as well as future directions in both clinical application and research.

**Key words:** *Pediatric cardiology. Genomics. Molecular biology.*

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## INTRODUCTION

Congenital heart disease (CHD), cardiomyopathy, and arrhythmias are common causes of mortality and morbidity in infants and children, particularly during

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## Cardiología pediátrica en la era de la genómica

A pesar de que las enfermedades cardíacas congénitas, las miocardiopatías, las arritmias y las enfermedades cardíacas adquiridas son causas frecuentes de mortalidad y morbilidad en niños de todas las edades, los mecanismos básicos que subyacen a muchas de estas enfermedades cardiovasculares pediátricas siguen sin esclarecerse. Los grandes avances en la tecnología genética molecular empiezan ahora a aplicarse en la cardiología pediátrica a partir del uso de mapas cromosómicos y de la identificación de los genes involucrados tanto en la etiología primaria como en los factores de riesgo significativos para el desarrollo de anomalías cardíacas y vasculares. Esta revisión se centrará en la información que se ha obtenido hasta el momento a partir de análisis genéticos moleculares en el diagnóstico, el tratamiento y la comprensión general de la patogenia de la enfermedad cardiovascular pediátrica, examinando tanto los defectos cardíacos congénitos/heredados más prevalentes, las arritmias y las miocardiopatías, como las alteraciones esporádicas y adquiridas. Además, se examinará el arsenal terapéutico disponible en cardiología pediátrica en relación con el análisis molecular y genético, haciendo especial hincapié en los usos actuales de los métodos de diagnóstico molecular. Estos métodos incluyen los «microarrays», los mapas genéticos, la proteómica y las tecnologías transgénicas y de células madre. Finalmente, se analizarán las direcciones futuras, tanto en la aplicación clínica como en la investigación.

**Palabras clave:** *Cardiología pediátrica. Genoma. Biología molecular.*

the perinatal period. Cardiovascular abnormalities represent the most common class of birth defect affecting 1 in every 100 infants each year. The high incidence of cardiovascular defects in infants and children presently represents an enormous burden and cost borne by the families, health-care providers and society at large.

While our understanding of pathology has grown rapidly in recent years, the basic underlying mechanisms of many specific pediatric cardiovascular diseases remain largely obscure. Given the manifold technical

## ABBREVIATIONS

ACE: angiotensin-converting enzyme.  
ARVD: arrhythmogenic right ventricular dysplasia.  
AVSD: atrioventricular septal defect.  
BMD: Becker muscular dystrophy.  
CHD: congenital heart disease.  
CVB: coxsackieviruses group B.  
DCM: dilated cardiomyopathy.  
DHPLC: denaturing high performance liquid chromatography.  
DMD: Duchenne muscular dystrophy.  
DMPK: myotonin protein kinase.  
ETC: electron transport chain.  
FAO: fatty acid oxidation.  
FISH: fluorescence in situ hybridization.  
FRDA: Friedreich's ataxia.  
HCM: hypertrophic cardiomyopathy.  
HERG: human ether-a-go-go related syndrome.  
HOS: Holt-Oram syndrome.  
JAG1: jagged-1.  
KCNE1: potassium channel, voltage-gated, Isk-related subfamily member 1.  
KCNE2: potassium channel, voltage-gated, Isk-related subfamily member 2.  
KVLQT1: potassium voltage-gated long QT syndrome 1 channel.  
LQTS: long QT syndrome.  
MBP: bone morphogenetic protein.  
MELAS: mitochondrial myopathy, encephalopathy, lactic acidosis and stroke like episodes.  
MERRF: myoclonic epilepsy and ragged red fibers.  
 $\alpha$ -MHC:  $\alpha$ -myosin heavy chain.  
 $\beta$ -MHC:  $\beta$ -myosin heavy chain.  
PCR: polymerase chain reaction.  
PDA: patent ductus arteriosus/Char syndrome.  
PKCepsilon: protein kinase cepsilon.  
RFLP: restriction fragment length polymorphism.  
RYR2: ryanodine receptor 2.  
SCN5A: sodium channel, voltage-gated, type V,  $\alpha$ -polypeptide.  
SR: sarcomeric reticulum.  
SSCP: single strand conformation polymorphism.  
TBX1: T-box 1 transcription factor.  
TBX5: T-box 5 transcription factor.  
TCA: citric acid cycle.  
TFAP2B: transcription factor of the AP-2 family.  
tRNA: transfer RNA.

breakthroughs associated with the sequencing of the human genome, research has begun to provide an increased understanding of specific molecular defects and to identify the specific "players" that contribute to the cardiac disorders, many involved with anomalies in the development of the heart. While the clinical applicability of these molecular techniques show great promise in the diagnosis, management and treatment of pediatric heart disease, their present use in the clinical setting has been generally limited in part because of the high costs and resources involved, and in part due to the complexities posed by genetic heterogeneity.<sup>1</sup>

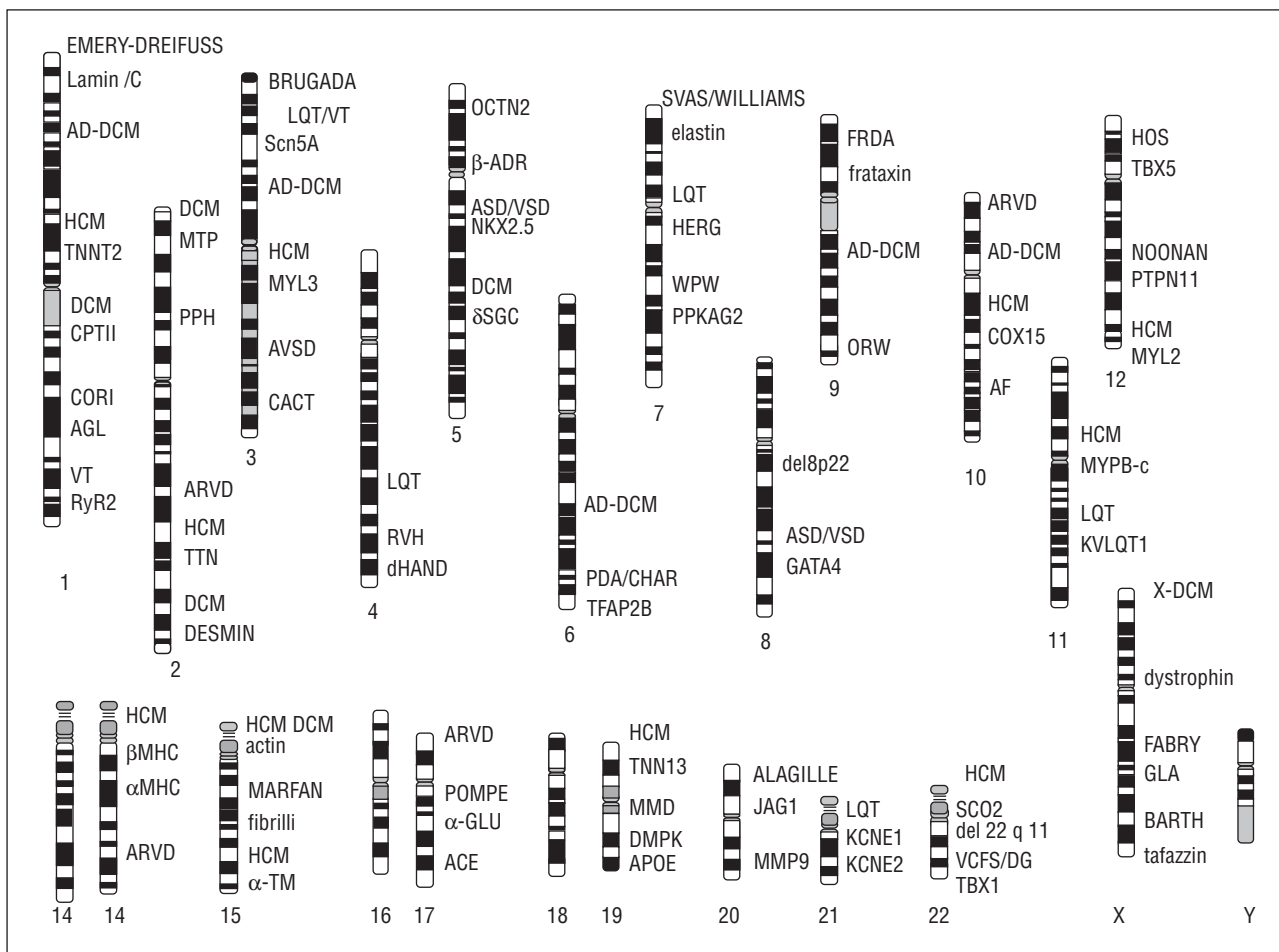
This review will focus on information provided by molecular and genetic analysis in the diagnosis, treatment and overall understanding of pediatric cardiovascular disease pathogenesis, addressing both the more prevalent congenital/inherited heart defects as well as sporadic and acquired disorders. A discussion of arrhythmias and cardiomyopathies in prenatal, neonatal and child patients from a molecular point of view will also be presented. Additionally, a survey of the pediatric cardiologist's armamentarium with regards to molecular and genetic analysis will be discussed highlighting the current use of molecular diagnostic methods including microarray, proteomic, transgenic and stem cell technologies as well as future directions in both clinical application and research.

## MOLECULAR GENETICS: DEFECTS LEADING TO CARDIAC DISORDERS

Recent advances in molecular genetics have revealed that specific genetic and molecular factors are linked to congenital heart disease and cardiac arrhythmias allowing their identification on the human chromosome map (Figure 1) and providing a valuable opportunity for improved genetic diagnostics and future gene therapy.

### Congenital Heart Diseases

Single gene mutations have been implicated in the pathogenesis of a variety of congenital heart defects (Table 1) and evidence suggests that these mutations (more common than previously thought) are present in a broad spectrum of genes involved in cardiac structure and function. The level of cardiac-specificity for these mutations is highly variable. Many single-gene mutations associated syndromes have neuromuscular and systemic presentation associated with cardiac involvement (e.g. Friedreich ataxia, Duchenne muscular dystrophy). A wide range of cardiac defects results from these genetic mutations including abnormalities in electro-physiological function (e.g. conduction defects and arrhythmias), extracellular matrix proteins, enzymes and membrane transporters involved in fatty



**Fig. 1.** Human chromosomal map of inherited pediatric cardiovascular disorders. Disorders and affected genes are localized on ideograms of each of the human chromosomes. These include: ACE, angiotensin-converting enzyme; AD-DCM, autosomal dominant dilated cardiomyopathy; AF, familial atrial fibrillation; AGL, glycogen-debranching enzyme; APOE, apolipoprotein E; ARVD, arrhythmogenic right ventricular dysplasia; AVSD, atrioventricular septal defect;  $\alpha$ -GLU,  $\alpha$ -glucosidase;  $\alpha$ MHC,  $\alpha$ -myosin heavy chain;  $\alpha$ -TM,  $\alpha$ -tropomyosin;  $\beta$ -ADR,  $\beta$ -adrenergic receptor;  $\beta$ -MHC,  $\beta$ -myosin heavy chain; CACT, carnitine-acylcarnitine translocase; CPTII, carnitine palmitoyltransferase II;  $\delta$ -SGC,  $\delta$ -sarcoglycan; DCM, dilated cardiomyopathy; DMPK, myotonin protein kinase; FRDA, Friedreich's ataxia;  $\alpha$ -GLA,  $\alpha$ -galactosidase; HCM, hypertrophic cardiomyopathy; HERG, human ether-a-go-go related syndrome; HOS, Holt-Oram syndrome; JAG1, jagged-1; KCNE1, potassium channel, voltage-gated, Isk-related subfamily member 1; KCNE2, potassium channel, voltage-gated, Isk-related subfamily member 2; KVLQT1, potassium voltage-gated long QT syndrome 1 channel; LQT, long QT syndrome; MMD, myotonic muscular dystrophy; MMP9, matrix metalloproteinase 9; MTP, mitochondrial trifunctional protein; MYBPC, myosin-binding protein C; MYL2, regulatory ventricular myosin light chain; MYL3, essential ventricular myosin light chain; OCTN2, organic cation carnitine transporter 2; ORW, Osler-Rendu-Weber syndrome; PDA, patent ductus arteriosus/Char syndrome; PPH, primary pulmonary hypertension; PPKAG2, AMP-activated protein kinase gamma 2; PTPN11, protein tyrosine phosphatase; SHP-2; RYR2, ryanodine receptor 2; RVH, right ventricular hypoplasia; SCO2, synthesis of cytochrome c oxidase (COX assembly protein); SCN5A, sodium channel, voltage-gated, type V,  $\alpha$ -polypeptide; SVAS, supraaortic stenosis; TBX1, T-box 1 transcription factor; TBX5, T-box 5 transcription factor; TFAP2B, transcription factor of the AP-2 family; TNNT2, cardiac troponin T; TNN13, cardiac troponin I; VCFS/DG, velocardiofacial syndrome/DiGeorge; VT, ventricular tachycardia; WPW, Wolff-Parkinson-White syndrome; X-DCM, X-linked dilated cardiomyopathy.

acid and mitochondrial biosynthesis, cardiac oxidative phosphorylation metabolism, sarcomeric structural and contractile proteins and nuclear transcription factors which govern myocardial gene expression and developmental programming. Pleiotropic cardiac malfor-

mations can result from discrete mutations in specific nuclear transcription factors, proteins recognized as playing key regulatory roles during heart development and morphogenesis.<sup>2-4</sup> Factors such as GATA4, NKX2.5, dHAND, TFAP2, and TBX5 are among the

TABLE 1. Inborn Errors Causing Congenital Heart Defects\*

Genes Affected (Loci)	Cardiac Phenotype/(Syndrome)	Reference
<i>Channelopathies/electro-physiology</i>		
Cardiac voltage-gated sodium channel-subunit (SCN5A)	Arrhythmia, ventricular tachycardia and fibrillation SD (Long QT and Brugada)	22, 23
HERG (KCNH2)	Cardiac arrhythmias, SD (Long QT)	23
MinK (KCNE1)	Cardiac arrhythmias, SD (Long QT)	23
MiRP-1 (KCNE2)	Cardiac arrhythmias, SD (Long QT)	23
KVLQT1 (KCNQ1)	Cardiac arrhythmias, SD (Long QT)	23
Nuclear envelope protein (lamin A/C)	Conduction defects, muscular dystrophy (Emery-Dreifuss)	25
Cardiac ryanodine receptor (RyR2)	Ventricular tachycardia	24
<i>Vascular extracellular matrix proteins</i>		
Fibrillin-1 (FBN-1)	Mitral or aortic valve regurgitation, SD (Marfan)	27
Elastin	Aortic and systemic arterial stenoses (Williams)	28
<i>Transcription factors</i>		
GATA4	Cardiac septal defects	5
TBX1	DiGeorge/velocardiofacial 17	
TBX5	Holt-Oram	9
CSX/NKX2.5	ASD/VSD A-V block	4, 6
dHAND	Right ventricle hypoplasia	3
TFAP2	Patent ductus arteriosus (Char)	8
<i>Signaling proteins</i>		
Protein tyrosine phosphatase SHP-2 (PTPN11)	Conduction defects	11, 12
Jagged 1 (Jag1)	Pulmonary stenosis (Noonan and LEOPARD) Pulmonary artery stenosis Tetralogy of Fallot (Alagille)	10
Myotonin protein kinase (DMPK)	Arrhythmias and conduction defects (myotonic muscular dystrophy)	14

\*SD indicates sudden death; ASD, atrial septal defect; VSD, ventricular septal defect.

earliest transcription factors expressed in the developing heart and are crucial in the activation of cardiac-specific genes. Mutations in each of these genes results in severe cardiac abnormalities including cardiac septal defects (GATA4), conduction defects (NKX2.5), right ventricular hypoplasia (dHAND), patent ductus arteriosus in Char syndrome (TFAP2B), and Holt-Oram syndrome (TBX5) underscoring the critical role played by the disruption of early heart development and morphogenesis in the genesis of congenital heart defects.<sup>5-9</sup>

Genetic defects in proteins involved in the multiple signaling pathways which modulate cell proliferation, migration and differentiation in early cardiac development have also been identified. Mutations in *Jag1* have been identified in kindred studies in association with Alagille syndrome, a complex autosomal-dominant disorder presenting with congenital

heart defects including pulmonary artery stenosis and Tetralogy of Fallot.<sup>10</sup> *Jag1* encodes a ligand that binds the Notch receptor, an evolutionarily conserved signaling pathway involved in cell fate specification. Mutations in PTPN11 encoding a protein tyrosine-phosphatase (SHP-2) have been proposed to play a role in the pathogenesis of Noonan syndrome characterized by conduction defects, pulmonary stenosis and hypertrophic cardiomyopathy<sup>11</sup> and have been also recently implicated in the pathogenesis of LEOPARD syndrome, which likely represents an allelic disorder.<sup>12</sup>

In addition to point mutations in coding regions of specific genes, a number of inherited neuromuscular disorders referred to as Triplet Repeat Syndromes including Friedreich ataxia and myotonic muscular dystrophy are caused by expanded repeats of trinucleotide sequences within specific genes e.g. frataxin (FRDA)



and myotonin protein kinase (DMPK) respectively.<sup>13-14</sup> Affected individuals exhibit severe cardiac abnormalities including cardiomyopathy, cardiac arrhythmias and conduction defects. In both disorders, the severity of the clinical phenotype correlates with the number of nucleotide repeats, i.e. >200 repeats of GAA are found in affected individuals with Friedreich ataxia, while >50 copies of CTG in cases with myotonic muscular dystrophy.

Large chromosomal deletions have also been implicated in developmental/structural malformations of the heart including conotruncal abnormalities, atrioventricular canal defects, ventricular and atrial septal defects.<sup>15,16</sup> Cardiac outflow tract defects are a manifestation of the complex genetic disorder velocardiofacial syndrome/DiGeorge syndrome, also termed CATCH-22. Most patients are hemizygous for a 1.5- to 3.0-Mb deleted region of chromosome 22 (22q11), suspected to be critical for normal pharyngeal arch development, which contains over 30 genes; the del22q11 deletion is a relatively common event occurring in approximately 1 in 4000 live births. A gene *TBX1* derived from the central area of the deleted region has been identified as the critical factor in the development of this congenital defect.<sup>17</sup> *TBX1*, a member of a phylogenetically conserved family of genes that share a common DNA-binding domain (i.e. the T-box) encodes a transcription factor involved in the regulation of cardiac development; reduction in *TBX1* expression (which occurs in the deleted hemizygous state) often referred to as haploinsufficiency impacts greatly on the early gene expression involved in cardiac morphogenesis. Other chromosomal microdeletions have been reported in association with congenital heart defects (e.g. 8p) and it is possible that some may have been previously overlooked due to smaller size and chromosomal location.<sup>18</sup> Newer molecular cytogenetics techniques with high resolution such as fluorescence *in situ* hybridization (FISH) are currently routinely utilized to confirm the clinical diagnoses of chromosomal damage such as chromosomal microdeletions and small translocations.

It is important to note that such large genetic deletions are commonly associated with a wide spectrum of clinical features in addition to cardiac involvement. Extra-cardiac malformations are commonly associated with congenital heart defects and have been estimated at over 30% of cases. These chromosomal anomalies are more prevalent in patients with cardiac anomalies than in the general population. In addition, while neonatal cardiac malformations resulting from trisomies 13, 18, and 21 (Down syndrome), as well as the monosomy XO (Turner syndrome) are well-recognized, the precise molecular basis by which the gene dosage imbalance in these patients causes the cardiac phenotype has not yet been elucidated.

## Arrhythmia and Sudden Death

Cardiac arrhythmias are a frequent complication of pediatric heart disease and can be a primary cause of sudden cardiac death. Mutations in specific genes encoding cardiac ion channels have been identified as a risk factor in the pathogenesis of lethal and nonlethal arrhythmias. Mutations in *SCN5A*, a gene which encodes sodium channels responsible for initiating action potentials, is associated with prolongation of the QT interval or long QT syndrome (LQTS) which causes a predisposition to syncope and sudden cardiac death<sup>19,20</sup>. The phenotypic hallmark of LQTS is abnormal ventricular repolarization and can result in idiopathic ventricular fibrillation, ventricular tachycardia, cardiac conduction defects and Brugada syndrome<sup>21,22</sup>. Mutations in 4 other genes (i.e. *HERG*, *KCNE1*, *KCNE2*, *KVLQT1*) involved in the formation of cardiac potassium channels have also been associated with the onset of LQTS.<sup>23</sup> These defects are characterized by significant genetic heterogeneity with over 30 mutations identified in 40 families.

Mutations in an assortment of membrane transporters operating at cellular loci other than the myocardial plasma membrane have been implicated in atrioventricular conduction defects broadening substantially the concept of cardiac channelopathies. Missense mutations in the ryanodine-receptor calcium release channel (*RyR2*) involved in excitation-contraction coupling of the sarcomere have been identified in stress-induced calcium overload in myocytes leading to ventricular tachycardia.<sup>24</sup> Also, discrete mutations in the lamin A/C gene encoding the nuclear envelope proteins lamin A and lamin C are present in individuals affected with the autosomal dominant form of Emery Dreifuss muscular dystrophy who display familial partial lipodystrophy, dilated cardiomyopathy (DCM), atrioventricular conduction defects and atrial fibrillation.<sup>25</sup>

In addition, the accumulation of intermediary metabolites of fatty acids, such as long-chain acylcarnitines, can result in severe cardiac arrhythmias and conduction defects in the neonate. Inborn errors of fatty acid oxidation (e.g. carnitine palmitoyltransferase II, mitochondrial trifunctional protein and carnitine-acylcarnitine translocase deficiencies) have been reported in unexplained sudden infant death or near-misses, and in infants with conduction defects or ventricular tachycardia<sup>26</sup>.

## Vasculopathies

Molecular genetic defects have been identified as underlying autosomal dominant vasculopathies including Marfan syndrome, supravalvular aortic stenosis, and Williams' syndrome indicating the critical role that microfibrils and extracellular matrix defects play in the pathophysiology of these disorders.<sup>27,28</sup> Marfan

TABLE 2. Genetic Defects in Pediatric Cardiomyopathy\*

Gene Product Affected (Gene Locus)	Cardiac Phenotype	Reference
<i>Structural/contractile proteins</i>		
$\beta$ -myosin heavy chain ( $\beta$ -MHC)	HCM	33
$\alpha$ -myosin heavy chain ( $\alpha$ -MHC)	HCM	32
Essential myosin light chain (MYL3)	HCM	82
Regulatory myosin light chain (MYL2)	HCM	82
Actin	DCM, HCM	34, 41
$\alpha$ -tropomyosin ( $\alpha$ -TM)	HCM	30
Cardiac troponin T (TNNT2)	HCM	30
Cardiac troponin I (TNNI3)	HCM	31
Desmin	DCM	42
$\delta$ -sarcoglycan ( $\delta$ -SGC)	DCM	43
Myosin binding protein c (MYBPC)	HCM	29
Titin (TTN)	HCM	35
Dystrophin	DCM (Duchenne and Becker muscular dystrophy)	39, 44
X-linked DCM		
<i>Metabolism and bioenergetics</i>		
Mitochondrial trifunctional protein (MTP)	Cardiac arrhythmias, SD, DCM	83
Carnitine palmitoyl transferase II (CPTII)	Cardiac arrhythmias, SD, CM	84
Carnitine-acylcarnitine translocase deficiency (CACT)	Cardiac arrhythmias, SD, CM	85
Carnitine transport (OCTN2)	HCM, DCM	86, 87
Tafazzin (G4.5)	DCM (Barth)	41
Mitochondrial Fe <sup>++</sup> metabolism (frataxin)	HCM (Friedreich ataxia)	13, 51
Very-long-chain acyl-CoA dehydrogenase (VLCAD)	HCM, SD	88
Lysosomal $\alpha$ -glucosidase (acid maltase/glycogen storage)	Ventricular pre-excitation HCM (Pompe's)	89
Glycogen-debranching enzyme (AGL)	HCM (Cori's)	90
$\alpha$ -galactosidase (GLA)	HCM (Fabry's)	91
Mitochondrial heme metabolism (COX15)	Early-onset fatal HCM	36
$\delta$ -2 subunit of AMP-activated protein kinase (AMPK)	HCM, conduction defects (Wolff-Parkinson-White)	38
<i>Mitochondrial DNA</i>		
tRNA <sup>Leu</sup> , tRNA <sup>Ala</sup> , tRNA <sup>Ile</sup> , tRNA <sup>Gly</sup>	HCM (MELAS, MERRF)	37, 48
ATPase6	HCM (Leigh)	92
Sporadic mtDNA deletions	HCM, conduction defects (KSS)	60

\*HCM indicates hypertrophy cardiomyopathy; DCM, dilated cardiomyopathy; SD, sudden death.

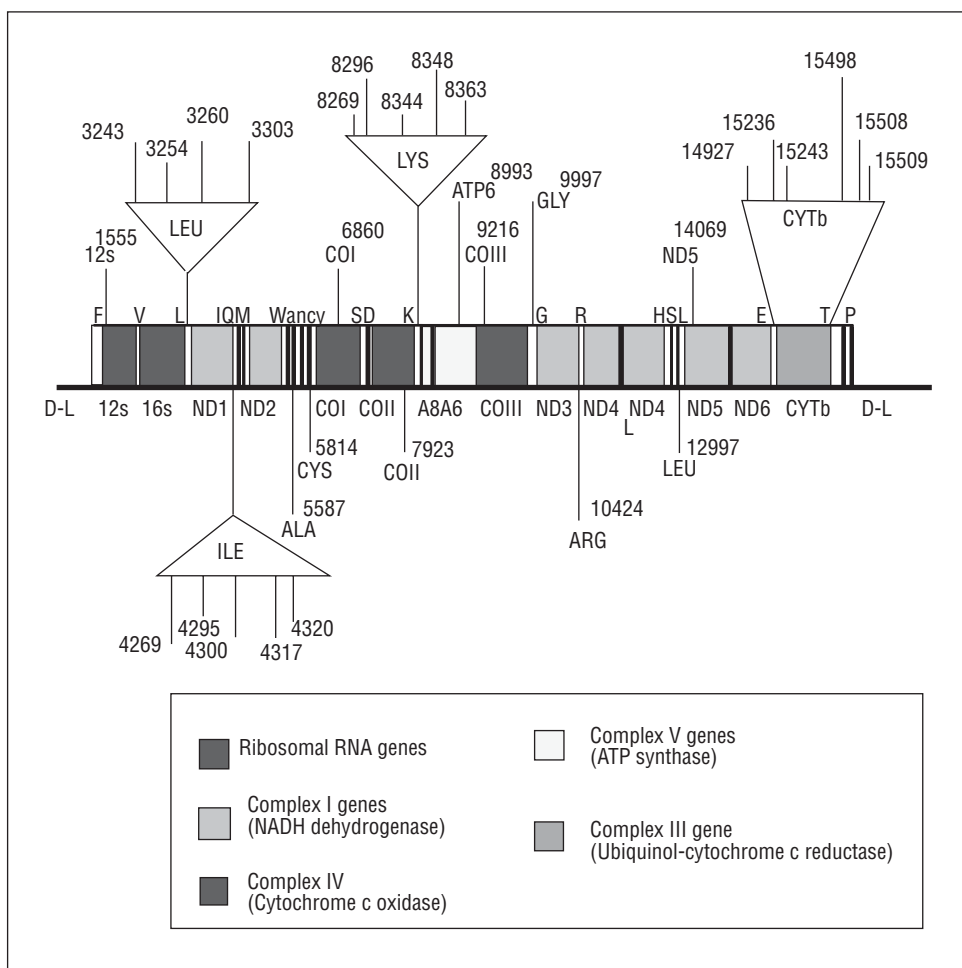
syndrome is characterized by abnormalities in skeletal, ocular and cardiovascular systems which can lead to premature death primarily due to progressive dilation of the aortic root with fatal aortic dissection or aortic insufficiency, and is associated with high neonatal mortality due to polyvalvular involvement with subsequent severe congestive heart failure. Most cases of Marfan syndrome with cardiovascular disease have mutations in the fibrillin gene, with most families having a distinct mutation. Fibrillin is a constituent of a multiprotein complex (including elastin) present in the microfibril component of the large-vessel vascular wall. Mutations in the gene encoding a component of the extracellular matrix (i.e. elastin) are responsible

for supravalvular aortic stenosis that features obstruction resulting from discrete narrowing of the ascending aorta and Williams syndrome presenting with stenoses of systemic and/or pulmonary arteries.

### Cardiomyopathy

Mutations causing human cardiomyopathies have also been identified in a broad spectrum of nuclear genes encoding myocardial contractile proteins and structural proteins, enzymes involved in glycogen storage (Pompe's and Cori's diseases) and mucopolysaccharide degradation (Fabry disease), lipid metabolism (fatty acid  $\beta$  oxidation and carnitine deficiency) and in both

**Fig. 2.** Pathogenic mitochondrial DNA mutations associated with pediatric cardiac disease. A linear representation of the circular 16 569 basepair human mtDNA molecule showing the relative location of all 13 protein-encoding genes (ND1-ND6, COI-COIII, cytb, and ATP6 and ATP8), 22 tRNAs identified by their cognate amino acid using single letter code (F, V, L, Y, Q, M, W, A, N, C, Y, S, D, K, G, R, H, S, L, E, T, P), the 2 rRNA genes (12s and 16s) and the non-coding D-loop region (D-L). The nucleotide location and genes containing pathogenic mtDNA mutations associated with pediatric cardiac disease are indicated as shown.



nuclear and mitochondrial DNA (mtDNA) encoded genes essential for cardiac energy production (as shown in Table 2). While both DCM and hypertrophic cardiomyopathy (HCM) have been shown to occur in the young and can have genetic/familial components, HCM has been more fully characterized since it represents the most frequent cause of sudden cardiac death in children and adolescents<sup>1</sup>. Most cases of familial HCM exhibit a pattern of autosomal dominant transmission (the exception being those cases of pathogenic mtDNA mutations which are maternally inherited). Mutations causing HCM have been found in over 10 genes encoding different sarcomeric proteins including  $\beta$ -myosin heavy chain ( $\beta$ -MHC),  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC), myosin-binding protein C, cardiac troponin T and troponin I,  $\alpha$ -tropomyosin, essential and regulatory myosin light chains, titin and cardiac  $\alpha$ -actin.<sup>29-40</sup> In addition, specific defects in genes involved in mitochondrial heme and Fe<sup>++</sup> metabolism (e.g. nuclear encoded frataxin and COX15),<sup>13,36</sup> and in mito-

chondrial bioenergetics (e.g. mtDNA encoded tRNAs and ATPase6)<sup>37</sup> have been detected in patients with HCM (albeit more rarely than the sarcomeric mutations). In addition, mutations in the regulatory subunit of AMP-activated protein kinase (AMPK), a key sensor and mediator in cellular energy metabolism, have been reported in a subset of cases of HCM.<sup>38</sup> Taken together, these findings suggest that cardiac mitochondrial energy depletion can be an underlying cause of HCM in some patients rather than depressed sarcomeric contraction and could be helpful in understanding a number of clinical observations in HCM such as its heterogeneity, variable onset and severity and hypertrophic asymmetry.

Currently, approximately 30% of all cases of DCM are estimated to be inherited, while 70% appear to be sporadic. Genes for X-linked familial DCM (dystrophin, G4.5) have been identified<sup>39,40</sup> and several genes for the autosomal dominant form of DCM (actin, desmin, lamin A/C,  $\delta$ -sarcoglycan) have been reported.<sup>41-43</sup>

In cases of X-linked DCM attributed to a gene defect in dystrophin (a large cytoskeletal protein associated with the sarcolemma) the defect in dystrophin is manifested only in cardiac myocytes; the site of the mutation is primarily located in the promoter regulatory region of the dystrophin gene which is consistent with its tissue-specific expression.<sup>44</sup> Mutations in the dystrophin gene can also lead to both Duchenne (DMD) and Becker (BMD) muscular dystrophies affecting both skeletal and cardiac function.<sup>39</sup> Typically, patients with the more severe DMD lack detectable dystrophin protein in skeletal muscles, caused by the presence of either deletion mutations in dystrophin alleles that disrupt the translational reading frame or specific point mutations that create stop codons. Male patients with X-linked DCM (due to dystrophin defect) tend to be asymptomatic in early childhood and develop syncope and rapidly progressive congestive heart failure in late adolescence; affected females generally display a later onset. In DMD, skeletal muscle weakness is present at an early age (3-6 years). Subsequently, more than 30% of the patients develop signs of cardiac dysfunction by age 14 and virtually all DMD patients develop DCM by age 18.

Barth syndrome, an X-linked cardioskeletal myopathy with neutropenia and dilated cardiomyopathy often presents in infancy. The protein tafazzin responsible for Barth syndrome is encoded by the G4.5 gene and belongs to a family of acyltransferases involved in phospholipid synthesis.<sup>40,45</sup> In patients harboring the G4.5 mutation, saturated fatty acid levels increase while unsaturated fatty acid and cardiolipin levels are markedly reduced affecting cardiac membrane fluidity and function. Arrhythmogenic right ventricular dysplasia (ARVD) is an autosomal dominant form of cardiomyopathy characterized by progressive degeneration of the right ventricular myocardium, arrhythmias and increased risk of sudden death which has been recently mapped by linkage analysis to loci on several chromosomes including 2, 10, 14 and 17<sup>46</sup> although the precise genetic defect has not yet been determined.

Cardiomyopathy in neonates and children can also be due to underlying deficiencies of energy production due to both genetic and sporadic defects at a wide spectrum of loci.<sup>37,47</sup> Genetic disorders in energy metabolism leading to either specific fatty acid oxidation/carnitine deficiencies or OXPHOS abnormalities can also result in either a DCM or HCM phenotype and their increased recognition has led to the suggestion of an entity termed mitochondrial cardiomyopathy characterized by abnormal cardiac mitochondria either in number, structure or function. A number of mitochondrial cardiomyopathies have been described in association with neurological disorders such as MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy and ragged red fibers) and Leigh syndromes<sup>37,48</sup> with

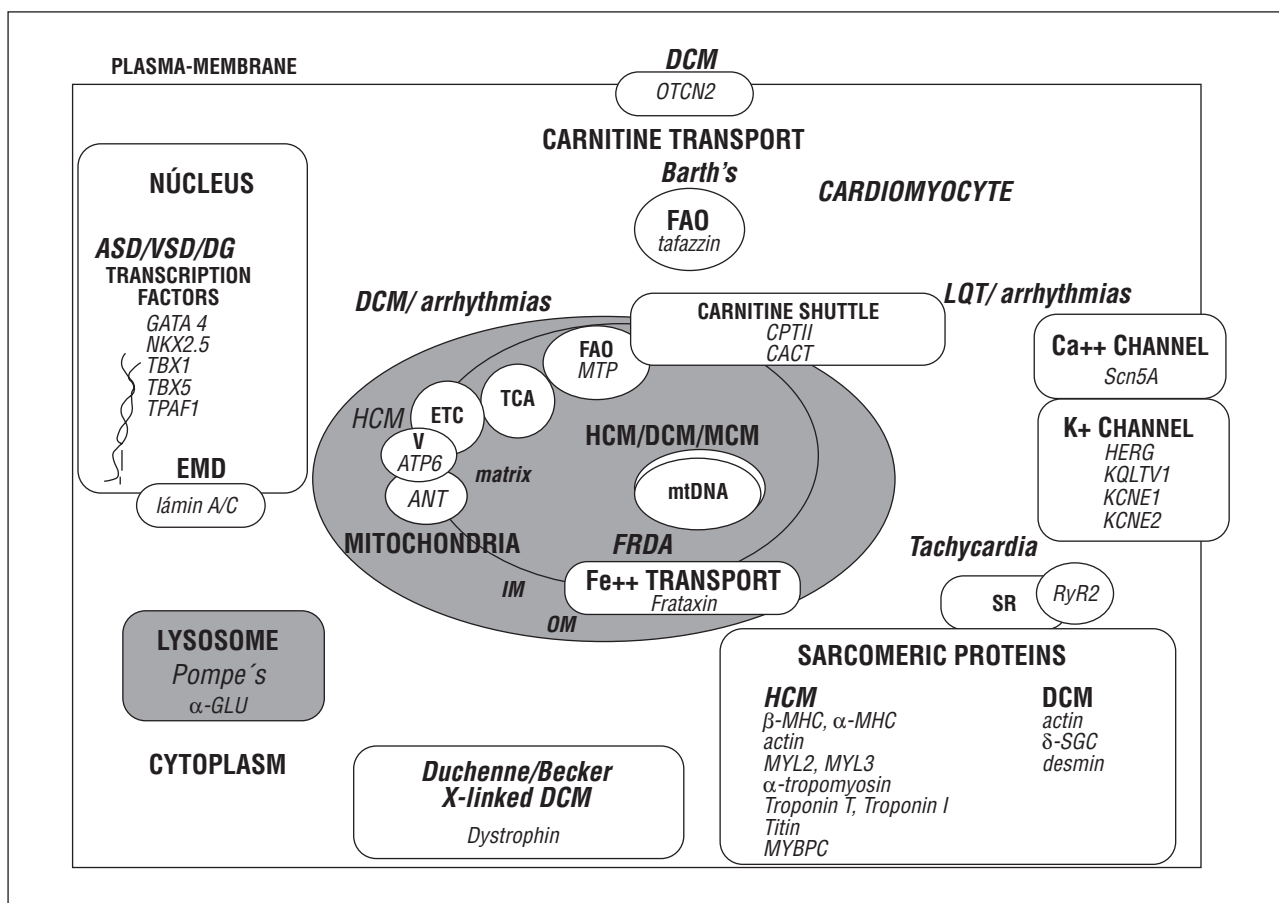
specific pathogenic mutations identified in several mtDNA genes needed for mitochondrial function (listed in Figure 2) and more recently in nuclear genes involved in the assembly of mitochondrial respiratory complexes.<sup>36,49</sup> These disorders may present early in childhood, while others manifest themselves later. Mitochondrial enzyme and DNA defects have also been noted in cases of fatal infantile cardiomyopathy.<sup>50-52</sup> Molecular studies of patients with either HCM or DCM have resulted in the further identification of novel pathogenic mtDNA mutations prevalent in cardiac tissues.<sup>53,54</sup>

Mitochondrial cardiomyopathy can also occur in a sporadic fashion. Agents that cause damage to cardiac mitochondria and mtDNA such as adriamycin and alcohol can result in cardiomyopathy.<sup>55,56</sup> Somatic generated (sporadic) deletion mutations in cardiac mtDNA have been shown to increase during myocardial ischemia<sup>57</sup> and their increased presence (although in low overall abundance) has been reported in the cardiomyopathic heart which may arise from increased oxidative stress.<sup>58,59</sup> In addition, Kearns-Sayre syndrome, a neuromuscular disorder with atrio-ventricular conduction defects and cardiomyopathy is commonly associated with abundant large-scale mtDNA deletions whose generation is thought to arise spontaneously since they are rarely detected in mothers or siblings.<sup>60</sup>

In contrast, DCM associated with multiple, abundant mtDNA deletions have been reported as a distinct phenotype due to genetic defects either dominantly or recessively inherited.<sup>61</sup> Linkage analysis in families with dominantly-inherited mtDNA deletions have identified in affected individuals any of several specific mutations in proteins that participate in mtDNA replication (e.g. mtDNA polymerase  $\gamma$  gene and the Twinkle gene, a putative mitochondrial helicase) and in mitochondrial nucleotide metabolism (e.g. adenine nucleotide translocator).<sup>62</sup>

Depletion in cardiac mtDNA levels has also been reported in young children with isolated cardiomyopathy, either DCM and HCM.<sup>54,63</sup> Recently several nuclear loci have been identified likely responsible for mtDNA depletion, a phenotype which is rarely assessed. Autosomal-recessive mutations in factors which play a role in mitochondrial nucleotide metabolism e.g. thymidine kinase 2, thymidine phosphorylase and deoxyguanosine kinase have been identified in a subset of patients (and their families) with mtDNA depletion.<sup>62</sup> In addition, depletion of cardiac mtDNA levels can be specifically induced by zidovudine (AZT), which inhibits both the viral DNA polymerase and mitochondrial DNA polymerase thereby stopping mtDNA replication.<sup>64</sup> However, recent studies do not support a role of zidovudine in association with the development of cardiomyopathy in infants or children treated with AZT.<sup>65</sup>





**Fig. 3.** Subcellular location within the cardiomyocyte of the defective gene-products found in pediatric cardiac disease. Organelles e.g. nucleus, lysosome, mitochondria, sarcomere/sarcomeric reticulum (SR) and plasma membrane sites are shown in larger bold, italic font. The compartments of the mitochondria are indicated including matrix, outer membrane (OM) and inner membrane (IM) Specific cardiac defects associated with specific cardiomyocyte-localized defects are indicated in bold, italic font including atrial and ventricular septal defect (ASD/VSD), DiGeorge syndrome (DG), Barth's syndrome, Pompe's disease, Emery-Dreifuss muscular dystrophy (EMD), Duchenne/Becker muscular dystrophy, Friedreich ataxia (FRDA), tachycardia, LQT syndrome, arrhythmias, HCM, DCM and MCM (hypertrophic, dilated and mitochondrial cardiomyopathy). Functional pathways are indicated in smaller plain font including transcription factors, membrane transporters, shuttles and channels, sarcomeric proteins, fatty acid oxidation (FAO), citric acid cycle (TCA), electron transport chain (ETC) with associated complex V (V) and adenine nucleotide translocator (ANT). Specific proteins/gene products involved are displayed in smallest plain italic font. Abbreviations used are described in Figure 1.

### Cellular Targets in Cardiac Disease

It should be clear from the foregoing discussion, that genetic defects resulting in cardiac structural and functional abnormalities target a diverse set of molecules within the cardiomyocyte as well as outside the cardiomyocyte (e.g. vasculopathies). Specific targets can be localized in a variety of subcellular compartments including the nucleus, mitochondria, lysosome, cytoplasm, endoplasmic reticulum and plasma membrane as depicted in Figure 3. In addition, these molecules (whether receptors, enzymes, channels or kinases) often play multiple roles in several interacting signaling pathways involved in the cell cycle, metabolic, developmental and physiological transi-

tions. The close intersection and communication signaling between these diverse pathways has made the unraveling of cardiac events highly informative although arguably more complex, and has important ramifications for therapeutic treatments focused on any specific target.

### Molecular Diagnosis Techniques, Limitations, and Advances

Many of the nuclear gene defects implicated in cardiomyopathies were originally mapped by linkage analyses in affected families, allowing the subsequent identification of candidate genes (and mutant alleles)

TABLE 3. Genes With Polymorphic Variants Contributing to Cardiovascular Disease\*

Gene Affected (Loci)	Normal Function	Associated Cardiac Phenotype	Drug Response Effected
ATP-cassette binding protein (ABC or MDR)	Lipid transport	Coronary artery disease	Digoxin
Angiotensin converting enzyme (ACE)	Renin-angiotensin regulator	Coronary artery disease	Angiotensin converting enzyme inhibitors
$\beta$ -adrenergic receptor (ADR $\beta$ -2)	Neurohormone Receptor	Congestive heart failure	$\beta$ -2 adrenergic agonists
Apolipoprotein E (APOE)	Lipid transport	Coronary artery disease	Statin
Cholesterol ester transport protein (CETP)	Lipid transport	coronary artery disease	Statin
minK related protein (KCNE2/MiRP1)	Potassium channel	Antibiotic-induced cardiac arrhythmia	Clarithromycin
Plasminogen-activator inhibitor type 1 (PAI-1)	Intravascular fibrinolysis	Myocardial infarction	ND
Stromelysin-1 (MMP-3)	Matrix metallo-proteinase	Myocardial infarction angina	ND
Thrombospondin (TSP-1)	Angiogenesis inhibitor	Premature coronary artery disease	NS
Nuclear transcription factor (NFATC4)	Transcription factor	Cardiac hypertrophy	ND
Interleukin-6 (IL-6)	Inflammatory mediator	Myocardial infarction	ND
Endothelin receptor A (ETA)	Vaso-regulator	Idiopathic DCM	ND

\*ND indicates not determined.

by positional cloning and subsequent nucleotide sequence analysis. A variety of molecular techniques including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) have been used in screening defective alleles from the proband and family members to establish inheritance patterns. In most cases, detection of novel mutation by itself is an immense undertaking involving the comprehensive analysis of large and multiple coding regions (exons) of one if not more candidate genes. Moreover, in the relatively well-characterized cases of familial HCM gene screening, the consensus experience has been that each specific HCM-causing mutation is rare challenging the view of common mutations since most families have “private” or novel mutations. Nevertheless, correlation of the clinical course and prognosis with specific mutations has proved informative; for instance, specific  $\beta$ -MHC mutations in HCM are associated with a high incidence of sudden death whereas other mutations are associated with a better prognosis. Recent advances in the speed and sensitivity of mutation detection by applying high throughput analytical techniques such as denaturing high performance liquid chromatography (DHPLC) or high-throughput capillary array electrophoresis should improve further the use of molecular genetic analysis in clinical and preclinical diagnosis and to provide specific targeted treatments in pediatric cardiac disorders. Moreover, in the near future, the availability of gene chip technology will allow automated and rapid

screening of mtDNA and nuclear gene mutations.

While modern imaging techniques are helpful in defining cardiac phenotypes in affected children, both genetic heterogeneity and intrafamilial variability have made the precise molecular elucidation of many cardiac defects, as well as the correlation of genotype with cardiac phenotype particularly difficult. These difficulties may arise from the involvement of undefined multifactorial or polygenic factors which can contribute to the expression of specific cardiac gene defect(s), as well as to a variety of epigenetic or acquired influences. Progress is gradually being made in defining these polygenic and epigenetic factors, some of which are also amenable to molecular analysis.

Increasing evidence supports the thesis that the genetic background in which deleterious mutations occur can significantly modulate their phenotypic expression. The presence of modifier genes in the genetic background which influence the phenotypic expression and severity of pathogenic HCM genes has been well established.<sup>66</sup> The identification of modifier genes which will markedly improve the elucidation of genetic risk factors has been assisted by large-scale genome-wide approaches to identify polymorphic variants correlated with disease severity. Single nucleotide polymorphism association studies have identified several candidate modifier genes for various cardiac disorders. A number of specific genetic polymorphisms have been found in association with myocardial infarction, coronary artery disease and hypertrophic cardiomyopathy as shown in Table 3. With the increased

cataloging of single nucleotide polymorphisms either alone or within a larger chromosomal region (haplotypes) in available shared databases, these modifier loci can be evaluated for their effects in predisposing to specific cardiac defects and may impact on the choice of diagnostic and treatment options.

By utilizing a genome-wide analysis of cardiovascular disorders, a larger net can be cast for detecting associated disease-related mutations. Recent methodological advances have made it possible to simultaneously assess the entire profile of expressed genes in affected myocardium requiring only very limited amount tissue, a significant fact in neonates and children. Foremost among these methods is gene expression profiling using DNA microarrays. Microarrays are artificially-constructed DNA grids in which each element of the grid acts as a probe for a specific RNA. Gene expression by microarray analysis has proved to be a useful tool in establishing pathophysiological features of a disease by comprehensive evaluation of which genes are increased and which are decreased in expression, and can be applied in both clinical diagnosis and in evaluating patients' response to therapy.

The association of defective genes with cardiac disorder uncovered by genomic analysis needs to be followed by proteomic analysis to establish the function and pathophysiological role played by the mutant protein and to reveal interacting modulators. Once the implicated genes and their gene-products have been fully identified, sequence and subsequent bioinformatic analysis can be employed to identify common structural and functional motifs and homologies with known proteins. The potentially significant functional interaction of proteins (which can be an important determinant of the cardiac phenotype) can be further determined by yeast two-hybrid analysis. This approach has been productive in establishing that mutant titin proteins (derived from patients with HCM) had reduced binding affinities for other specific sarcomeric proteins (e.g.  $\alpha$ -actinin), as well as in characterizing the synergistic interactions of transcription factors NKX2.5 and TBX5 in early cardiac development.<sup>67</sup>

### Transgenic Analysis

The role of a particular gene and its product in determining specific cardiac phenotypes can be further confirmed *in vivo* by using targeted gene ablation or gene "knock-outs," most commonly introduced in transgenic mice. For instance, mice containing null alleles for genes involved in mitochondrial fatty acid oxidation (e.g. mitochondrial trifunctional protein), mitochondrial DNA transcription and bioenergetics (e.g. mitochondrial transcription factor A) and in the gene encoding mitochondrial frataxin rapidly develop severe cardiac dysfunction and DCM consistent with

the clinical findings of cardiomyopathy associated with specific mutations in a variety of loci involved in mitochondrial bioenergetic function.<sup>68-70</sup> This technique has also proved highly informative in establishing the critical role of TBX1 in the etiology of DiGeorge/velocardiofacial syndrome. Mice heterozygous for a single null allele of TBX1 exhibit a high incidence of cardiac outflow tract anomalies as well as other developmental abnormalities common to DiGeorge syndrome.<sup>71</sup>

### Fetal Abnormalities: Molecular Analysis

Three-dimensional reconstruction of heart defects by using ultrasound, x-ray or MRI has dramatically improved the diagnosis and the therapeutic strategies of cardiac diseases. Most forms of congenital heart disease can be detected *in utero*. Following the diagnosis of congenital heart disease, further evaluation for extracardiac anomalies and chromosomal abnormalities is recommended since these are found in up to 62% and 38% respectively of cases. Counseling based on the prenatal evaluation can provide realistic information about the incidence, diagnosis, and prognosis of the fetal heart defects. Prenatal diagnosis of congenital heart malformations and their molecular correlates (e.g. microdeletions of 22q11 in DiGeorge syndrome and 7q in Williams syndrome), detectable by cytogenetic and molecular techniques subsequent to amniocentesis, has proved to be a critical adjunct in the management of life-threatening malformations of the neonate such as transposition of the great arteries and hypoplastic left heart syndrome

### Acquired Cardiac Diseases in Children

Acquired cardiac diseases in neonates and children include Kawasaki disease, acute and chronic rheumatic heart disease, infective endocarditis and myocarditis. The use of molecular genetic technology has been applied in a limited fashion in their analysis and could provide improved clinical diagnosis. Kawasaki disease, an acute self-limited vasculitis of infancy and early childhood, is the leading cause of acquired heart disease in children in the United States and Japan.<sup>72</sup> Its etiology remains unknown, and extensive molecular analysis has thus far been unable to detect viral or bacterial involvement. If untreated, 25% of children develop aneurysms of the main coronary arteries. Since treatment is generally effective only if administered within the first 10 days of illness (to prevent coronary artery involvement) it poses a diagnostic challenge for the pediatric cardiologist who must distinguish Kawasaki disease from other diseases within a relatively limited timeframe. While intravascular ultrasound has the promise of improving assessment of coronary arteries, molecular markers

of the disease potentially identifiable by microarray analysis could prove a valuable asset in confirming the diagnosis.

Immunological and molecular analysis have implicated the presence of viral induction (most frequently involving coxsackieviruses group B (CVB) and aberrant autoimmune responses in the pathogenesis of pediatric myocarditis which in some cases may evolve into DCM. Recent molecular studies using the polymerase chain reaction have also identified adenovirus in addition to enterovirus in the myocardium of children with myocarditis and DCM.<sup>73</sup> In addition, although the precise pathogenic mechanism of streptococcal-induced rheumatic fever and rheumatic heart disease has not yet been fully elucidated, molecular analysis has provided significant insight into critical auto-immune aspects of the disease, and further gene linkage/association analysis may provide key information about genetic factors involved in host susceptibility. Molecular data may also prove useful in devising strategies for the management of cardiovascular abnormalities associated with acquired infections such as the pulmonary hypertension that can present with HIV infection.<sup>74</sup>

### Pharmacogenomics and Cardioprotection

Understanding pediatric cardiovascular disease at the genomic level may allow for more effective stratification of patient subclasses and targeted and optimized patient-specific therapy.

The related fields of pharmacogenomics and pharmacogenetics hold the promise of improved drug development and the tailoring of drug therapy based on the individual's ability to metabolize drugs which are determined only in part by age, and influenced by disease, environmental factors (e.g. diet), concurrent medications and variant genetic factors specifying the transport, metabolism and targets of the drug. For example, a subset of the single-nucleotide polymorphisms identified in human genes e.g. beta adrenergic receptor, and angiotensin-converting enzyme (ACE) have been associated with substantial changes in the metabolism or effects of medications used in the treatment of cardiovascular disease, and may be informative in predicting the clinical response (Table 3).<sup>75</sup> Individualizing therapy may be particularly critical in establishing drug dosages and efficacies in children with cardiovascular disease, a population for which pharmacokinetics has proven to be poorly defined and often unpredictable. Both immunological and genetic phenotyping of pediatric patients can provide a more effective therapeutic strategy, either by inhibiting or stimulating specific responses.

A growing body of evidence has established that cardioprotection can be elicited by either ischemic preconditioning or by pharmacological means (e.g. ni-

corandil and diazoxide) and can potentially be harnessed as a strategy for organ and tissue protection in ischemic heart disease and hypoxic insult, albeit at this time there is limited data concerning the cardioprotection responses in infants and children. Extensive work in several animal models has established that the molecular basis of the cardioprotection mechanism(s) involves a network of signal transduction pathways mediated by cell-surface receptors, the activation and subcellular translocation of specific protein kinases (e.g. PKCepsilon, P38 MAP kinase, and JUN kinase) and the opening of both sarcolemmal and mitochondrial  $K_{ATP}$  channels.<sup>76</sup> Infants with cyanotic heart defects and hypoxia were found to have activated myocardial protein kinase levels of PKCepsilon, P38 MAP kinase, and JUN kinase not present in either infants with acyanotic defects or normal subjects indicating that the cardioprotective signal transduction pathway is at least partially operative in hypoxic infants.<sup>77</sup> Cardioprotection associated with stress-protein and mitochondrial signaling has also been demonstrated with brief periods of hypothermia prior to a prolonged ischemic insult<sup>78</sup> and may be involved in the effective clinical management of junctional ectopic tachycardia by hypothermia.<sup>79</sup> Further research in this area could reveal potential target molecules (e.g. receptor, signaling kinase or channel) for highly-specific pharmacological intervention. However, as a cautionary note, this may take time and an increased understanding of the network of interacting pathways. Despite the recent achievements in the identification of precise genetic and signaling defects causing cardiac arrhythmia, the development of effective drugs (e.g. specific ion channel blockers), that can substantially reduce the mortality associated with severe arrhythmic disorders, has shown strikingly little success thus far underscoring the complex cardiac circuitry, multiple causal, genotypic and risk factors involved in evolving disease phenotypes.<sup>80</sup>

### Future Frontiers

Despite significant advances in the diagnosis and treatment of cardiac diseases in children, many fundamental questions remain unanswered regarding their basic underlying mechanisms and pathophysiology. Breakthroughs in molecular genetic technology have just begun to be applied in studies of cardiovascular disease allowing chromosomal mapping and the identification of many genes involved in both the primary etiology and also as significant risk factors in the development of these anomalies. The following areas of research appear quite promising:

1. Since our understanding of cardiac and vascular development is still in its infancy, the further identification of novel genes involved in cardiac organogene-



sis and vascular development will serve as an important foundation for our understanding how specific congenital gene defects generate their cardiac phenotypes. Bioinformatic methods can be employed to search existing databases with the routinely used reverse genetics techniques, allowing subsequent cloning of novel genes/cDNAs of interest followed by the characterization of spatial-temporal patterns of specific gene expression in the developing embryo (using *in situ* hybridization).

2. The mechanisms governing the early specification of cardiac chambers in the developing heart tube have not yet been precisely delineated but are thought to involve novel cell-to-cell signaling, amongst migrating cells, as well as the triggering of chamber-specific gene expression programs, mediated by specific transcription factors and growth factors such as Bone morphogenetic protein (MBP). Future areas of study will focus on elucidating the role of signaling molecules (e.g. WNT) using conditional gene knock-outs (in a variety of genetic backgrounds) and accessing their interaction with critical transcription factors such as dHAND, NKX2.5, GATA4, and TBX. Similar approaches may also prove informative in probing the origins of the cardiac conduction system, and in deciphering the role of signaling systems as participants in vascular formation in endothelial cells, focusing on the interaction of VEGF, angiopoietin, TGF, and the Notch pathway.

3. Another critical area of research is the identification of molecular regulators that control cardiomyocyte proliferation. Cardiomyocytes are mitotically active during embryogenesis and generally cease proliferation shortly after birth. Understanding the molecular basis of cardiomyocyte proliferation could greatly impact on our clinical attempts to repair damaged heart tissue. Mechanism of cell growth regulation may be investigated by careful comparison of comprehensive gene expression profiles of embryonic and postnatal myocytes, as well as by the generation of myocyte cell culture lines with the capacity to respond to proliferative inducers. Alternatively, cellular transplantation is a mechanism with which to augment myocyte number in diseased or ischemia damaged hearts. Interestingly, a recent study demonstrated that a subpopulation of adult cardiac stem cells injected into an ischemic heart were able to fully reconstitute well-differentiated myocardium, differentiating into both cardiomyocytes and new blood vessels.<sup>81</sup> Nevertheless, new research efforts will be necessary to further define the optimal conditions necessary for cardiomyocyte differentiation and proliferation and for the fully functional integration of stem cells in the myocardium, as well as to investigate the ability of transplanted stem cells to repair defects in children's hearts. It will be critical to learn whether severe cardiac abnormalities such as cardiomyopathies (e.g.

DCM), Kawasaki disease with myocardial damage and ARVD can be rectified by stem-cell transplantation.

Insight into the cardiovascular consequences of abnormal gene function and expression should ultimately impact on the development of targeted therapeutic strategies and disease management for children with congenital and acquired heart disorders and may replace less effective treatment modalities directed solely at rectifying structural cardiac defects and temporal improvement of function.

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